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Proteins of Milk

INTRODUCTION

Proteins are of great importance in biochemistry, since they form a large proportion of the food and the body tissues of man and other animals. Research on proteins is extremely difficult because of the large size and complex structure of their molecules and aggregates, their insolubility in most common solvents, their usually noncrystalline form, and the readiness with which they undergo decomposition or other chemical change when attempts are made to isolate and purify them or to subject them to the usual methods of characterization. Proteins may be defined as substances composed principally of amino acids chemically combined. Carbon, hydrogen, nitrogen, oxygen, sulfur, and, in a few instances, phosphorus are the elements present in proteins. Cow's milk contains approximately 3.55% protein, or, as distributed, 3.00% casein and 0.55% whey proteins.

Useful reviews of the chemistry of milk proteins have been published by McMeekin and Polis[127] in 1949 and by McMeekin[124] in 1953.

NOMENCLATURE

In discussing the proteins of milk, it is necessary to distinguish between the proteins *in* milk and those obtained *from* milk by various chemical and physical fractionation procedures. Because of the ease with which casein can be isolated from milk, the earliest subdivision of milk proteins was to casein and whey proteins. However, this implies that casein exists in milk in the form of the same entity as it does in the isolated state, which is not true when the casein is precipitated by acid, as is customary. The casein is in the form of a complex or micelle, consisting of calcium caseinate plus phosphate, additional calcium, magnesium, and citrate. **Casein**

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may be defined as the protein precipitated by acidifying skimmilk to a pH value near 4.6. It is, in chemical language, hydrogen caseinate. The proteins remaining after casein has been removed from skimmilk are known as **whey proteins** or **milk serum proteins.** They have been fractionated by salting-out methods to produce a lactalbumin fraction and a lactoglobulin fraction.[39,152,187] The casein fraction, the lactalbumin fraction, and the lactoglobulin fraction were generally considered single chemical entities until Linderstrøm-Lang[118,119] in 1925, Cherbuliez and Schneider[30] in 1932, and Bugai[21] in 1935 reported the fractionation of casein, and Palmer[155] in 1934 reported further fractionation of the whey proteins. Since then research has resulted in the isolation of eight main components of milk proteins, several of which are apparently heterogeneous as indicated by electrophoretic measurements.

Subdivisions of milk serum proteins sometimes mentioned in milk protein literature[92] are **heat-labile milk serum proteins** and the **proteose-peptone fraction,** the first being the portion of the milk serum proteins rendered acid-precipitable at pH 4.6–4.7 by previous heat treatment of the milk or whey, the second being the portion not rendered precipitable by these means. **Commercial lactalbumin** is a mixture of the heat-labile, acid-precipitable milk serum proteins. The so-called classical **lactalbumin fraction** is the portion of the milk serum proteins soluble in neutral half-saturated ammonium sulfate solution or neutral saturated magnesium sulfate solution. In a classification advocated by Rowland,[180] **lactalbumin** is the portion of the heat-labile milk serum protein which is soluble in saturated magnesium sulfate solution. The classical **lactoglobulin fraction** is the portion of the milk serum proteins insoluble in neutral half-saturated ammonium sulfate solution or saturated neutral magnesium sulfate solution. In the Rowland classification, **lactoglobulin** is the portion of the heat-labile milk serum proteins insoluble in saturated magnesium sulfate solution. A complex of proteins and enzymes adsorbed on the surface of the fat globules of milk is usually designated the **membrane proteins of the fat globules.** The eight components of the milk proteins that have been isolated with the aid of the electrophoretic method may be defined by their isoelectric points and by their electrophoretic mobilities. These and other identifying characteristics are listed in Table 21 which has been adapted from similar tables in the reports of Jenness *et al.*[92] and Brunner *et al.*[19]

TABLE 21

SOME PROPERTIES OF PROTEINS ISOLATED FROM MILK[a]

Protein	Reference to Method of Preparation	Approximate % of Skimmilk Protein[b]	Molecular Weight	Isoelectric Point[c]	Electrophoretic Mobility[d] at pH 8.6	Other Distinguishing Characteristics
α-Casein	80, 225	45–63	121,800[202]	4.1,[225] 4.7[79]	−6.7[79]	Contains 1% phosphorus. Consists of a mixture of proteins
β-Casein	80, 225	19–28	24,100[202]	4.5,[225] 4.9[79]	−3.1[79]	Contains 0.6% phosphorus
γ-Casein	80	3–7	30,600[144]	5.8–6.0[79]	−2.0[79]	Contains 0.1% phosphorus
β-Lactoglobulin	155	7–12	35,000[150]	5.1[170]	−5.1[170]	Consists of 2 forms, A and B, genetically determined[6]
α-Lactalbumin	58, 62	2–5	15,500[63]	5.1[101], 4.1–4.8[58]	−4.2[58]	Contains 7% tryptophan
Blood serum albumin	169	0.7–1.3	69,000[169]	4.7[169]	−6.7[169]	Apparently identical with albumin of bovine blood
Euglobulin	192, 193	0.8–1.7	180,000[192] 252,000[144]	6.0[192]	−1.8[192]	Contain antibodies. Contain hexose and hexosamine. Present in high concentration in colostrum
Pseudoglobulin	192, 193	0.6–1.4	180,000[192] 289,000[144]	5.6[192]	−2.2[192]	

[a] Adapted from the reports of Jenness *et al.*[92] and Brunner *et al.*[19]

[b] Values compiled from Rowland nitrogen distribution data, relative areas of electrophoretic patterns, and protein yield studies[80,92,106,179,180,188]; a proteose-peptone fraction amounting to 2–6% of the total protein is also present.

[c] Values represent points of zero electrophoretic movement or minimum solubility or both.

[d] Electrophoretic mobility (u) = $\times 10^{-5}$, cm^2, volts^{-1}, sec.$^{-1}$ obtained by the Tiselius moving boundary method at 2° in veronal buffer at pH 8.6, ionic strength = 0.1; measured from descending pattern.

DETERMINATION OF PROTEIN FRACTIONS

The separation of the proteins of skimmilk into the casein, lactalbumin, and lactoglobulin fractions,[39,152] that was employed for many years, was preparatory to the determination, characterization, and study of each of these fractions. The methods employed were precipitation of casein by acidification to its isoelectric point, and salting-out methods for the lactalbumin and lactoglobulin fractions. Rowland[180] devised an analytical scheme to determine these fractions, a fraction consisting of proteoses and peptones, and a fraction consisting of nonprotein nitrogen compounds. The results are usually expressed in terms of the percentage distribution of nitrogen among the five fractions. The preparation of samples for study is incidental. The following is an outline of the Rowland scheme.

Total N(I) is determined on the milk by the Kjeldahl procedure. Noncasein N(II) is determined by precipitating casein by a mixture of acetic acid and sodium acetate, filtering, and analyzing the filtrate for N. Nonprotein N(III) is determined by precipitating the total protein by means of 15% trichloroacetic acid, filtering, and analyzing the filtrate for N. Nonprotein N plus proteose-peptone N(IV) is determined by boiling an aliquot of the filtrate II, adjusting the reaction to pH 4.75, filtering, and analyzing the filtrate for N. Lactoglobulin N(V) is determined by adjusting an aliquot of the filtrate II to pH 6.8–7.2, saturating it with magnesium sulfate, filtering off the lactoglobulin fraction after several hours, washing it with saturated magnesium sulfate solution, and analyzing it for N. Then, casein N = I − II, lactalbumin N = II − (IV + V), lactoglobulin N = V, proteose-peptone N = IV − III, and nonprotein N = III. Rowland,[181] by use of his method, found the average nitrogen distribution in normal milk to be: 78.3% casein N; 9.1% lactalbumin N; 3.5% lactoglobulin N; 4.1% proteosepeptone N; and 5.0% nonprotein N.

The classical fractions obtained by the Rowland procedure were investigated electrophoretically by Larson and Rolleri.[106,179] The electrophoretic method not only reveals the complexity of the fractions produced by chemical partition but gives somewhat different results, particularly in the distribution of the albumin and globulin fractions. The distribution percentages shown in Table 21 were compiled from both types of study which, incidentally, agree in assigning 2 to 6% of total skimmilk protein to the proteose-peptones fraction.[92]

A new chemical partition method employing sodium sulfate at

controlled pH values for the preparation of fractions was developed by Aschaffenburg and Drewry.[7] This procedure gives results in substantial agreement with the electrophoretic data and, besides, permits the further differentiation of the classical albumin fraction into α-lactalbumin and β-lactoglobulin fractions.

The electrophoretic method when applied to casein itself by Mellander[132] showed that the protein was made up of at least three components and these were designated α-, β-, and γ-casein in the order of decreasing mobility in a phosphate buffer.

Another method which has yielded important information about the proteins in milk is ultracentrifugation. It is mentioned here primarily in connection with the names of the individual whey proteins, α-lactalbumin, β-lactoglobulin, and γ- or immune lactoglobulin. In the sedimentation diagrams of decalcified skimmilk described by Pedersen[159] the peaks were identified by Greek letters in the order of increasing sedimentation constants, the smallest whey protein being designated α-lactalbumin, the next larger β-lactoglobulin (see Cannan *et al.*[25]), and the largest γ-lactoglobulin. Peaks with even larger constants were ascribed to components of casein.

The components of the milk protein fractions will be dealt with as individual proteins in later sections of this chapter.

THE CASEINATE COMPLEX

The principal protein in milk is present in the form of a complex, existing as particles of macromolecular sizes, and consisting of calcium caseinate, components that can be obtained from milk as calcium phosphate, and magnesium and citrate ions. Whether calcium phosphate is present in the complex as an entity and whether it is bound to calcium caseinate by adsorption or by other bonds are questions still unanswered. The casein complex is considered in detail elsewhere in this volume.

CASEIN

Elementary Composition

In the early years of protein chemistry, the determination of the elementary composition of isolated proteins, as of other organic chemicals, was an important method of characterization. With the recognition that proteins are very large molecules, composed largely of the same amino acids, it became evident that differences in carbon and hydrogen content could not be great and determina-

tions of these elements are seldom made at present. Nevertheless, because casein was one of the first proteins to be prepared in purified form and because of their historical interest, some elementary analyses of purified, acid-precipitated casein are listed in Table 22. The determinations of phosphorus, sulfur, and nitrogen compare

TABLE 22

ELEMENTARY COMPOSITION (PER CENT) OF ACID-PRECIPITATED CASEIN

	Year	Carbon	Hydrogen	Nitrogen	Sulfur	Phosphorus	Nitrogen Factor
Hammarsten[72]	1883	53.0	7.05	15.65	0.76	0.85	6.39
Lehmann, Hempel[109]	1894	54.0	7.04	15.60	0.77	0.85	6.41
Tangl[208]	1908	52.7	6.81	15.65	0.83	0.88	6.39
Van Slyke[220,221]	1913	53.5	7.13	15.80	0.72	0.71	6.33
	1918					0.80	
		53.6	7.13	15.66	0.64	0.81	6.39
Mellander[133]	1947	53.1	6.95	15.65	0.81	0.85	6.39

favorably with more recent analyses. Estimations of these elements are still useful in the characterization of casein and other proteins since phosphorus is an uncommon constituent of proteins, sulfur provides a measure of total sulfur-containing amino acids present, and nitrogen percentage is a convenient, rough index of the content of protein in foods, feeds, and other biological materials. The nitrogen factors shown in Table 22 are simply 100 divided by the nitrogen percentages. It has been customary for many years to estimate the protein content of biological materials by determining their nitrogen content, usually by the Kjeldahl method, and multiplying by the factor 6.25 because purified proteins contain about 16% nitrogen. In the case of dairy products, the factor used is 6.38, this figure reflecting more accurately the nitrogen content of milk proteins.

Amino Acid Composition

Many important discoveries regarding proteins and their amino acid composition were made in the nineteenth and early twentieth centuries by investigators who used the readily prepared casein as a typical protein. For example, a few amino acids were discovered in hydrolyzates of casein and were recognized later to be common constituents of all proteins.[223] Casein was also widely used in the development of methods for the quantitative amino acid analysis of proteins. Actually, however, it was another milk protein, β-lactoglobulin, which was the first large protein to have its complete amino

acid composition established by Brand *et al.*[18] in 1945. A few years later casein and its main components were also analyzed in this manner[61]; the results are summarized in Table 23. Compilations of the many quantitative determinations of amino acids in casein and other milk proteins have been made by Block and Weiss[13] and Orr and Watt.[151]

TABLE 23

AMINO ACID COMPOSITION OF COW MILK PROTEINS
(Grams per 100 gm. protein)

Constituent	Casein[a]	α-Casein[a,b]	β-Casein[a]	γ-Casein[a]	β-Lactoglobulin[c]	α-Lactalbumin[d]	Blood Serum Albumin[e]	Immune Globulin[f]
Total N	15.63	15.53	15.33	15.40	15.60[18]	15.86	16.07	15.3–16.1
Total P	0.86	0.99	0.61	0.11	0.0	0.0	0.0	0.0
Total S	0.78	0.72[79]	0.86[79]	1.03[79]	1.60[18]	1.91[58]	1.94	1.00[192]
Glycine	2.0	2.3	1.6	1.5	1.4	3.2	1.8	5.2
Alanine	3.2	3.8	2.0	2.3	7.0	2.1	6.3	4.8
Valine	7.2	6.3	10.2	10.5	6.1	4.7	5.9	9.6
Leucine	9.2	7.9	11.6	12.0	15.5	11.5	12.3	9.6
Isoleucine	6.1	6.4	5.5	4.4	6.9	6.8	2.6	3.0
Proline	10.6	7.5	15.1	17.0	5.1	1.5	4.8	10.0
Phenylalanine	5.0	4.6	5.8	5.8	3.5	4.5	6.6	3.9
Tyrosine	6.3	8.1	3.2	3.7	3.7	5.4	5.1	6.7
Trytophan	1.7	2.2	0.83	1.2	2.7	7.0	0.58	2.7
Serine	6.3	6.3	6.8	5.5	4.0	4.8	4.2	11.5
Threonine	4.9	4.9	5.1	4.4	5.0	5.5	5.8	10.5
Cystine + cysteine	0.34	0.43	0.0	0.0	3.4	6.4	6.5	3.2
Methionine	2.8	2.5	3.4	4.1	3.2	0.95	0.81	0.9
Arginine	4.1	4.3	3.4	1.9	2.8	1.2	5.9	4.1
Histidine	3.1	2.9	3.1	3.7	1.6	2.9	4.0	2.1
Lysine	8.2	8.9	6.5	6.2	11.8	11.5	12.8	6.8
Aspartic acid	7.1	8.4	4.9	4.0	11.4	18.7	10.9	9.4
Glutamic acid	22.4	22.5	23.2	22.9	19.3	12.9	16.5	12.3
Amide N	1.6	1.6	1.6	1.6	1.1[18]	1.4	0.78	—

[a] Analytical results from Gordon *et al.*[59–61]
[b] More recent analyses of a different preparation of α-casein and of components of α-casein are reported by Hipp *et al.*[78]
[c] Results listed are for β-lactoglobulin AB[54] or for a 1:1 average of A and B.[167]
[d] Gordon and Ziegler.[63]
[e] Stein and Moore.[198]
[f] Amino acid values from Hansen and Carlson[74]; figures were compiled from analyses by Smith[19] and Hansen *et al.*[75] of eu- and pseudoglobulin from milk and colostrum.

Sulfur

Casein contains 0.78% sulfur; the amino acids cystine and methionine account for 0.09 and 0.69%, respectively, cysteine being absent.[98] When casein is fractionated, it is found that α-casein contains all the cystine in casein.[61] On fractionation of α-casein, most

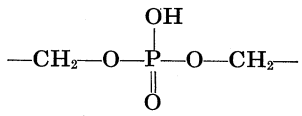
of the cystine is found in the α_3-casein component[78] or in Waugh's κ-casein.[227] The sulfur-containing amino acids of casein were suspected to be a source of the sulfhydryl, or mercapto, compounds produced in heated milk, but, since casein heated by itself does not produce sulfhydryl groups, the source is evidently not the casein.[95]

Phosphorus

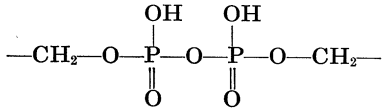
When casein is treated with proteolytic enzymes, rather large polypeptides, some of which contain phosphorus and resist further enzymatic degradation, are formed. It has been thought that these products, often called phosphopeptones, may play an important role in the nutrition of the young mammal. Thus, Mellander has suggested that phosphopeptones can combine with other nutrients, such as calcium and iron, in this way favoring absorption of these elements, and, furthermore, that during subsequent digestion a unique mixture of amino acids is provided at the proper time for optimal utilization of protein.[134]

In 1927, Posternak[171] isolated a phosphopeptone from tryptic digests of casein and found that it contained 5.9% phosphorus, 11.9% nitrogen, and glutamic and aspartic acids, serine and isoleucine. He believed that all of the phosphoric acid found had been linked to serine, presumably because serine was the only hydroxyamino acid present in the hydrolyzate. Independently, Rimington and Kay[174,176] by similar methods isolated a phosphopeptone containing 7.1% phosphorus and 10.1% nitrogen and came to the same conclusion regarding the linkage of phosphorus to hydroxyamino acids; however, the supposed isolation of hydroxyamino acids other than serine (hydroxyglutamic and hydroxyaminobutyric acids) in this work was shown later to be in error.[175] That some phosphoric acid is indeed bound to serine was proved by the isolation of phosphoserine from a weak acid hydrolyzate of casein by Lipmann[120] in 1933. At about the same time Levene and Hill hydrolyzed further a phosphopeptone from casein and isolated a dipeptide made up of phosphoserine and glutamic acid.[111] Twenty years later, in 1953, de Verdier[222] was able to prepare phosphothreonine from casein hydrolyzed by weak acid. From the preceding evidence and many other studies of a similar nature, it may be concluded that the phosphorus in casein is bound chiefly, if not entirely, in ester linkages with the hydroxyl groups of serine and threonine.

As to the nature of the ester linkages, Perlmann has postulated that not only O-monophosphate ester linkages, but also diester,



and pyrophosphate



linkages occur in casein and that the principal electrophoretic components of casein, α- and β-caseins, differ in types of linkage present.[160] However, there have been a considerable number of more recent investigations which indicate that phosphorus is bonded in the same way in whole casein, α-casein, and β-casein and that the bond is most likely the O-monophosphate ester linkage.[3,88,96,154,183]

Work on the isolation of phosphopeptides and phosphopeptones has continued to provide important information concerning the structure of casein. This research was facilitated by the fractionation of casein into its electrophoretic components and by the development of newer, more powerful methods of separating and characterizing the products of partial hydrolysis of the protein. For example, after partial acid hydrolysis of α-casein, Hipp *et al.* isolated phosphoserine, phosphoserylglutamic acid, phosphoserylalanine, and phosphoserylphosphoserine by ion-exchange chromatography.[83] Peterson *et al.* isolated and characterized a relatively large, electrophoretically homogeneous phosphopeptone from tryptic digests of β-casein. The phosphopeptone, with a molecular weight of about 3,000, consisted of 24 amino acid residues of 10 different amino acids and 5 phosphoric acid groups presumably attached to the 4 serine and 1 threonine residues present. Essentially, all the phosphorus of β-casein appears to be accounted for in this phosphopeptone.[166] A compilation of the results of other investigations of phosphopeptones prepared from α- and β-caseins has been made by Schormüller *et al.*[186]

Carbohydrate

The presence of small amounts of carbohydrate in casein has been detected by various investigators. For example, using the orcinol reaction, Sørensen and Haugaard[195] found highly purified, acid-precipitated casein to contain 0.31% hexose, believed to be galactose but later shown to be a mixture of hexose, hexosamine, and sialic acid.[38] Nitschmann *et al.*[149] demonstrated that a large pep-

de, which was split off from casein by the action of rennet, contained considerable amounts of galactose, glucosamine, and neuraminic acid; the peptide was called a glyco-macropeptide. It was shown subsequently that the carbohydrate is concentrated largely in the α-fraction of casein, and, more particularly, in the κ-portion of α-casein, from which the glycomacropeptide may be prepared.[2,94] Although quantitatively almost insignificant in casein, the carbohydrate moiety, as a constituent of κ-casein or of α_s-casein, may be uniquely important in the interactions which hold casein together.

Solubility

Casein is dissolved by aqueous solutions of acids, alkalies, and alkaline salts. The amount dissolved in a definite weight of solvent depends on the pH value of the solvent. In acid solutions of a pH on the acid side of the isoelectric point of casein, compounds are formed with the nonmetallic element or radical of the acid, and, in alkaline solutions, compounds are formed with the metal of the alkali or alkaline salt. Hence, such solutions are not solutions of casein in a strict sense. Solubility of casein, strictly speaking, is the solubility of chemically unaltered hydrogen caseinate, and hence is the solubility of casein in solutions at its isoelectric point. That point is usually considered to be pH 4.6,[138] but it is shifted by the presence of neutral salts in solution[102,139,196] and may be a point in a zone extending from pH 4.0 to pH 4.8, approximately. Much of the literature on solubility of casein does not state whether the determinations were carried out under isoelectric conditions and consequently must be accepted with reservations. Because the amount of casein dissolved by many solvents varies with the quantity of casein added to a definite quantity of solvent, many available data have limited usefulness. This is a necessary consequence of the fact that the casein preparations studied do not consist of a single molecular species, and therefore no true solubility constant for casein can be expected.

The solubility of casein in water at the isoelectric point has been reported as 0.05 gm./l at 5°C.,[161] and 0.11 gm./l at 25°C.[31] Propionic and acetic acids by themselves have no solvent action on casein, but, in the presence of aniline, phenol, glycine, or alanine, do have solvent power.[121] Formic, lactic, and pyruvic acids dissolve casein; dilution of such solutions with water causes precipitation of the casein. A lactic acid solution of casein may be diluted with ethanol, or a pyruvic acid solution with acetone, without causing precipitation. These solutions in the mixed solvents may be

diluted with water without becoming clouded. Casein dissolves in weak phosphoric acid[172] and in phenol.[33,67]

Casein is soluble in certain mixtures of water and organic solvents which separately do not have appreciable solvent action. From such solutions casein precipitates on dilution with water. Examples of these are 50% ethanol,[80,177] organic acid-ethanol-water and ethanol-benzene mixtures,[48] 50% pyridine,[113] and aqueous solutions of urea[22,67,80] chloral hydrate,[215] dihydroxybenzenes,[215] and pyrogallol.[230]

Aqueous solutions of sodium fluoride, sodium chloride, potassium chloride, barium chloride, calcium chloride, strontium chloride, magnesium chloride,[206] potassium bromide,[177] calcium bromide, calcium iodide, potassium sulfate, magnesium sulfate, disodium phosphate, ammonium acetate,[206] potassium acetate, potassium cyanide, sodium propionate, sodium butyrate, sodium valerate,[177] sodium tartrate,[48] sodium oxalate, ammonium thiocyanate,[177] potassium thiocyanate, lithium thiocyanate, calcium thiocyanate,[231] sodium benzenesulfonate, and sodium cymenesulfonate[123] have all been shown to have solvent action on casein, the proportion dissolved varying from a fraction of one per cent to nearly ten per cent.

Optical Rotation

Solutions of casein rotate polarized light in the levo direction. The specific rotation of casein in an 8.3% solution of orthophosphoric acid was reported by Rakuzin[172] to be $-86.6°$C. The same value was given for a solution in acetic acid. When the solvent was a 2% borax solution or a 2% hydrochloric acid solution containing 0.2% pepsin, $[\alpha]_D$ was found to be $-95.3°$C. Carpenter[26] found $[\alpha]_D^{20°} = -99°$C. for a 1.5% solution of casein in a phosphate buffer at pH 6.8; in more dilute solutions the specific rotation was increased. Zaykowsky[240] reported $[\alpha]_D^{18°} = -83.6°$C. for 2% casein dissolved in a 10% sodium acetate solution. Gould,[65] employing Zaykowsky's method, found $[\alpha]_D^{30°} = -81.7°$C. for Hammarsten casein; values for commercial caseins differed over the range of $-70°$ to $-90°$C. Golub and Pickett[53] showed that the optical rotation of casein did not vary between 20° and 60°C. but confirmed previous observations that it rose considerably as the pH was varied from 7 to 12. Hipp *et al.*[80] determined $[\alpha]_D^{25°}$ for casein and its main components in one per cent solutions in veronal buffer, pH 8.4, ionic strength 0.1; the values were $-105°$C. for unfractionated casein, $-87.4°$ to $-90.5°$C. for α-casein prepared by different methods, $-125°$ for β-casein, and $-132°$C. for γ-casein.

Racemization

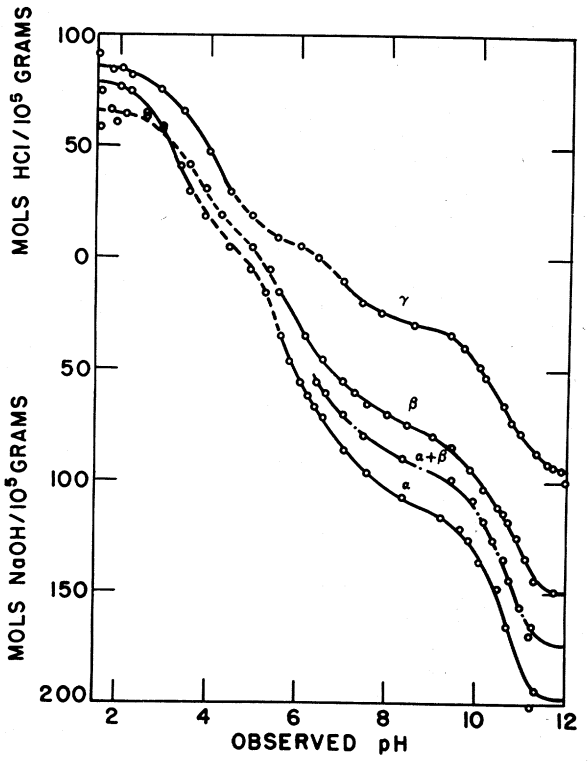
If casein is treated with dilute alkali and the mixture is incubated at 37°C. or at room temperature, the optical rotation steadily decreases in magnitude, approaching a value practically one-half that before treatment. Subsequent hydrolysis by means of acid causes the optical rotation to become zero. Dakin[41] proposed an explanation for this phenomenon based on a keto-enol tautomeric change in the peptide bonds of the protein but Csonka and Horn[40] considered that the lowered optical rotation of alkali-treated casein is due to a lower specific rotation of the hydrolytic products and that racemization is caused by the subsequent complete hydrolysis with acid and not by the incubation with alkali. It is evident, however, that the treatment of casein with alkali produces a unique change in the structure of casein other than hydrolysis since subsequent complete hydrolysis with acid produces racemic instead of the usual optically active amino acids. In any case the term "racemized casein" in the older literature refers to a partially degraded, alkali-treated casein which was not biologically available to the living organism.[42]

Combining Capacity

Much effort has been spent to determine the combining capacity of casein as an acid and as a base. Electrometric titration,[32,184] determination of the minimal amount of acid or base required to dissolve a given weight of casein,[34,161,162,177] and conductivity represent some of the methods employed. The results obtained for the combining capacity of casein have varied greatly depending on the method of preparing the casein and the method used to determine its combining capacity. Variations in the combining capacity of casein can in part be explained by the now known heterogeneity of the casein and its lability.

From the acid- and base-combining capacity of casein, Cohn and Berggren[32] calculated the number of its dissociating groups. They found that the number of dissociating groups could be correlated with the amino acid composition of the casein. More recently, Hipp *et al.*[82] have determined the acid- and base-binding capacities of the principal components of casein, namely α-, β-, and γ-casein, from their titration curves (Fig. 1).

By selecting the pH values where the ionic groups of the amino acid residues would be expected to dissociate, the number of ionizing groups were estimated from the titration curve. Thus at pH



After Hipp et al.[82]

FIG. 1. ACID BASE TITRATION CURVES FOR α-, β-, AND γ-CASEIN AND AN EQUAL MIXTURE OF α- AND β-CASEIN AT PROTEIN CONCENTRATION OF 1% 0.05 IONIC STRENGTH AND 25°C.

Ordinates are moles bound/10^5 gm. The dotted line is the pH region where, under the conditions of the experiment, all the protein was not soluble. For clarity, only a portion of the curve for the mixture of α- and β-casein is given.

6.35, carboxyl groups plus one equivalent of phosphoric acid are considered to be dissociated and were estimated by the equivalents of alkali combined at this pH. Similar estimates for the remaining dissociating groups in casein were made at other pH values. The estimates of the number of ionic groups in α-, β-, and γ-caseins arising from its amino acid residues were consistent with the number of ionic groups calculated to be present from the amino acid compositions of these proteins, as reported by Gordon *et al.*[59–61] and given in Table 23. The maximum acid- and base-combining capacities, as determined from the titration curves (Fig. 1) of α-, β-,

nd γ-caseins, were calculated to be 78, 66, and 85 moles of acid and 198, 150, and 96 moles of base per 10^5 gm., respectively. These values for the base-binding capacity are somewhat higher than the values of 176 and 128 moles per 10^5 gm. for α- and β-casein calculated from their amino acid compositions. However, the values determined for the base-combining capacity of α-, β-, and γ-casein by titration (Fig. 1) lead to a calculated value of 183 moles per 10^5 gm. of casein based on the composition of unfractionated casein being 16 parts α-, 4 parts β-, and 1 part γ-casein, which is in agreement with the value reported by Cohn and Berggren[34] for the base-combining capacity of several casein preparations.

TABLE 24

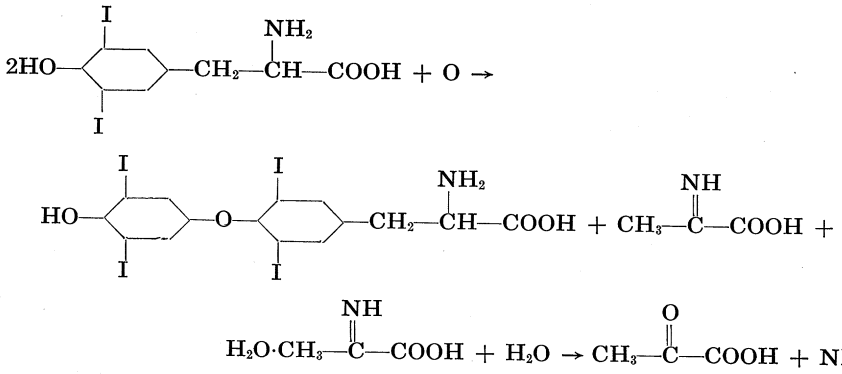
HALOGEN COMPOUNDS OF CASEIN

Investigator	Halogen Introduced	Reagents	Halogen Found, %
Habermann *et al.*[70]	Cl	KOH, $KClO_3$, HCl	13.3, 14.0
Panzer[156]	Cl	$KClO_3$, HCl	8.3
Salkowski[182]	Cl	$NaClO_3$, HCl	6.7, 6.8
Vandevelde[218]	Cl	CCl_4, Cl_2	32.5
Hopkins *et al.*[89]	Br	NH_4OH, Br_2	11.2
Vandevelde[217]	Br	CCl_4, Br_2	32.2, 35.0
Lépinois[110]	I	I_2, H_2O suspension	21.6
Liebrecht[117]	I	I_2, H_2O suspension	17.8, 8.7, 5.7
Masui[131]	I	NaOH, KI, I_2	10.8
Blum *et al.*[16]	I	$NaHCO_3$, I_2	7.5
Reineke *et al.*[173]	I	$NaHCO_3$, I_2	12.5, 8.8

Halogen Compounds

Casein, under some conditions, evidently forms definite chemical compounds with halogens; under other conditions the halogens appear to be merely adsorbed; and under still other conditions part of the halogen is chemically combined and part adsorbed. In Table 24 are listed the percentages of the halogens that have been introduced into the casein molecule to form what are apparently definite compounds. In the instances for which several percentages are given, it has been found that certain solvents can remove definite proportions of the halogen from the compounds containing the higher percentages, which indicates that part of the halogen is somewhat loosely bound. Lieben *et al.*[116] and Yaichnikov[239] treated dry casein with liquid bromine and, although a considerable percentage of bromine was taken up by the casein, no definite compounds could be identified and the bromine was subsequently slowly released. Yaichnikov[238] treated casein with iodine vapor and obtained similar results.

More recent research on the iodination of casein has been of considerable interest because of the formation of biologically active iodo-amino acids in the protein molecule. Reineke and Turner,[173] by treating casein with iodine in a solution of sodium bicarbonate, obtained an iodocasein containing as much as 12.5% iodine, but the proportion of iodine was decreased by dialysis, the highest percentage remaining being 8.78. A large proportion of the iodine that had combined was found to be attached to the tyrosine groups of the casein and part of this diiodotyrosine was oxidized to thyroxine. By raising the temperature to 70°C. and agitating vigorously, more of the diiodotyrosine was oxidized by atmospheric oxygen to thyroxine. Oxides of manganese also were effective oxidizing agents in this reaction. Hydrolysis of the product yielded free thyroxine, and some diiodotyrosine and monoiodotyrosine. They formulated the change as follows:



Pyruvic acid and ammonia were identified in the reaction products.

Michel and Volpert[142] prepared iodocasein in a somewhat similar manner with radioactive iodine and were able to demonstrate the presence of 3,5,3′-triiodothyronine also in enzymatic hydrolyzates of the iodinated protein. A reaction between monoiodo- and diiodotyrosine was postulated as leading to the synthesis of the triiodothyronine, a hormone associated with thyroxine in thyroglobulin.

Desaminocasein[46,77,112,114,146,184,190,199,200,201,232,236]

This desaminated casein is prepared by dispersing casein in water or in a pyridine-water mixture and treating with glacial acetic acid followed by a solution of sodium nitrite. The weight of the product is between 90 and 97.5% of that of the original casein. The percentage of nitrogen in the product may be decreased by as much as 0.7%, the ε-amino group of the lysine having been practically

entirely removed and the histidine, and possibly the arginine and tyrosine, having lost some nitrogen. Desaminocasein is digested and metabolized in the animal body, but it lacks the nutritional adequacy of casein, evidently because of the destruction of the lysine structure.

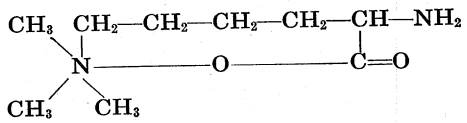
An attempt to improve casein fibers by desamination has been reported by Peterson and McDowell.[163]

Nitrocasein

Von Furth[51] nitrated casein at room temperature, with the addition of urea to avoid the effect of nitrous acid on the reaction. The product was bright yellow in color, soluble in water and alkalies, and was precipitated from solution by acids. It gave neither Millon's nor the lead acetate reaction. Nitrocasein may be fixed on cotton by steaming and thus becomes fast to rubbing, to soap, to chlorine, and to finishing treatments.[44] The xanthoprotein formed on adding nitric acid to casein without urea being present has characteristics slightly different from those of nitrocasein. Habermann and Ehrenfeld,[71] by heating casein with 13% nitric acid at 70°C., both hydrolyzed it and oxidized the hydrolytic products. They identified hydroxyglutaric acid as one of the resulting compounds.

Methylated Casein

Skraup and Krause[191] treated casein with methyl iodide and potassium hydroxide in absolute ethanol and obtained compounds which contained as much as 2.07% —O— CH_3 and 3.93% = N—CH_3 groups. Over five per cent iodine was introduced into the casein also. Geake and Nierenstein[52] employed diazomethane as a methylating agent for casein and increased the —O—CH_3 groups from approximately 0.50 to 2.08% and the = N—CH_3 groups from 0.88 to 2.28%. Herzig and Landsteiner,[76] using diazomethane, inincreased the —O—CH_3 groups in casein to 5.36% and the = N—CH_3 groups to 5.34%. Edlbacher[47] and Imai[91] used dimethyl sulfate and sodium hydroxide to methylate casein. Kapfhammer[97] showed that some of the methyl groups of methylated casein are attached to the ϵ-amino groups of lysine. He isolated from the products of hydrolysis an ϵ-betaine of lysine:



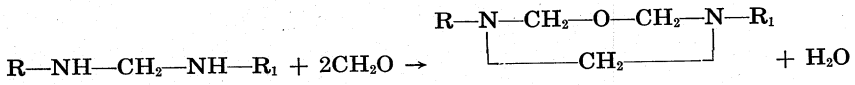
Acyl Derivatives

Troensegaard[213] acetylated casein by means of acetyl chloride and acetic acid. The acetyl casein contained 10.6% nitrogen and 46.3 acetyl groups per 100 atoms of nitrogen. Hydrogenation of the acetyl casein gave a mixture containing unidentified toxic substances.[214] Sandelin[184] dissolved casein in a mixture of equal parts of pyridine and water and acetylated with acetic anhydride. The product contained 14.52% nitrogen and had $[\alpha]_D^{18°} = -75.3°C$. Paracasein similarly treated gave a product containing 14.56% nitrogen and having $[\alpha]_D^{18°} = -74.4°C$. Schöberl and Krumey,[185] employing ketene, introduced 4.7% acetyl into casein. Kimura[100] obtained a benzoyl casein containing 15.02% nitrogen by treating casein with benzoyl chloride and sodium hydroxide. Benzoyl, lauryl, toluenesulfonyl, and trityl caseins have been made by treating casein in pyridine solution with the respective acid chlorides or anhydrides.[184,194] Phthalyl casein has been prepared by treating casein with phthalic anhydride and glacial acetic acid.[184] Acetylated, propionylated and butyrylated caseins were made by heating casein in the appropriate acid anhydride under various conditions and the products were evaluated as plastic molding powders[55]; also for the same purpose, higher fatty acid derivatives of casein containing approximately 20% of substituent groups ranging from caprylyl to stearoyl, were prepared by treating the protein in aqueous alkali with fatty acid chlorides.[56,57] By the action of benzoyl chloride on alkaline solutions of casein a series of benzoyl derivatives was made in connection with research on the vaporphase absorption of casein using these derivatives,[135] guanidated casein,[137] and glycine peptides[136] as model substances, Mellon and Hoover were able to conclude from their experiments that about 25% of the water absorbed by casein is absorbed by free amino groups, 45% by peptide bonds, and 10% by the guanidino groups of arginine.

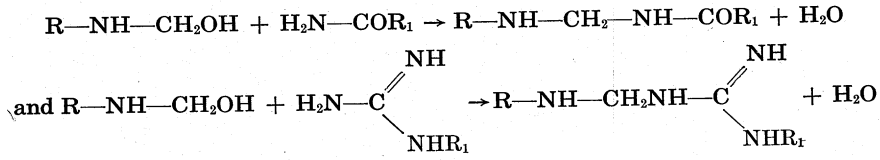
Formaldehyde Casein

The reaction of formaldehyde with casein is of practical as well as of theoretical interest, since formaldehyde is used as a hardening agent for plastic casein in industry. It is agreed that the free amino groups of casein are the points initially attacked, but several ideas have been advanced as to the reactions involved. Blum[15] believed that methylene caseins, $R—N=CH_2$ and $R–NH–CH_2–NH—R_1$, were formed with loss of water. Benedicenti[10] suggested that formaldehyde might be added, without elimination of water, to form a compound of the formula $R—NH—CH_2OH$. The prob-

ability that this reaction takes place is supported by the fact that much of the formaldehyde can be removed from formaldehyde casein by steam distillation, but the fact that heat treatment fixes part of the formaldehyde on the casein indicates that other reactions take place.[147,148,205] According to Wormell and Kaye,[237] formaldehyde, under neutral conditions, first becomes attached to each lysine side chain, and, in a second stage, cyclic compounds may be formed when two NH_2 groups have been linked by a methylene group in the first step:



Under acid conditions, amino and amide groups are linked through methylene groups. Fraenkel-Conrat and Olcott[49,50] have offered evidence that, in neutral or slightly acid solutions, CH_2O introduces $-CH_2-$ bridges between amino groups and reactive $=CH$ groups of phenolic and imidazole rings of proteins and that the hardening action of formaldehyde is not due to a primary reaction, such as that suggested by Benedicenti, but is due to a secondary reaction which transforms methylol, $-CH_2OH$, groups into cross-linking methylene bridges that link amino and primary amide or guanidyl groups:



Carbamido Casein

Hipp *et al.* treated casein with potassium cyanate to produce carbamido casein. In this reaction the basic amino groups of casein were replaced by weakly basic urea groups. Following hardening with formaldehyde the carbamido casein was tested as a plastic molding powder for the production of simple objects.[85]

Browning Reaction

Concentrated and dried forms of milk, when held for a considerable time in storage, gradually develop a brown color. The reaction involved has been studied by several investigators[36,107,108,143,157] who have found that, primarily at least, it is between the free amino groups of milk proteins and the aldehyde group of lactose. It is accelerated by increases in temperature, pH value, and water con-

tent of the protein-containing material, or in the relative humidity to which a solid protein is exposed. The reactions appear to be similar to those between casein and formaldehyde, not only in that the primary reaction involves aldehyde and free amino groups, but also in that there is a secondary reaction, which, in this instance, produces color and decreases the dispersibility of the protein. At first, lysine is the amino acid principally involved, but, later, arginine, histidine, methionine, and tyrosine are attacked. With the binding of the free amino groups of these essential amino acids, the biological value of milk protein is impaired. The literature on the chemistry of the browning reaction has been reviewed by Danehy and Pigman,[43] by Hodge[87] and, with particular reference to milk and dairy products, by Patton.[158] Haugaard and Tumerman[75a] have shown quantitative relationships for the aldose-amino interaction and suggested its significance in the incipient browning reaction.

Compounds of Sulfur and Casein

Uhl[216] treated an alkaline solution of casein with carbon disulfide and isolated a product of the reaction as a copper salt. He believed the reaction to be

$$R{-}NH_2 + CS_2 \rightarrow R{-}NH{-}\underset{\underset{S}{\|}}{C}{-}SH$$

However, since he used concentrations of alkali sufficient to hydrolyze the casein, the substance obtained probably consisted of amino thioacids. Gould,[66] using a minimal concentration of alkali, obtained a reaction between carbon disulfide and casein that produced large quantities of hydrogen sulfide. By precipitation with acid a substance was obtained from the solution having the same percentage of sulfur as the original casein. These results suggest the possibility that an isothiocyanate group was formed, which, on addition of acid, was hydrolyzed to the original amino group and a thiocarbonic acid or its anhydride:

$$R{-}NH_2 + CS_2 \rightarrow R{-}N{=}C{=}S + H_2S \text{ and } R{-}N{=}C{=}S + 2H_2O \rightarrow R{-}NH_2 + H_2CO_2S$$

Micheel *et al.*[140,141] have caused proteins to react with thioformamide and with dithioformic acid. Casein treated with dithioformic acid gave a thioformyl casein containing 2.48% sulfur. The reaction apparently involved the ϵ-NH_2 groups of the lysine residues.

Compounds of Metals and Casein

Compounds of casein with the alkali and alkaline earth metals are readily prepared by reaction with the hydroxide of the metal.

Compounds with other metals have been prepared by adding a solution of a soluble salt of the metal to a solution of an alkali caseinate and isolating the insoluble caseinate by filtration. Caseinates of such metals as copper, mercury, and silver have been prepared for use as antiseptics.

Decomposition Products

Products of various types have been prepared by subjecting casein to different intensities of oxidative or hydrolytic action. Most of such products are of little importance from the standpoint of the chemistry of milk. Some of these substances are described in the monograph on casein by Sutermeister and Browne.[203]

Precipitation of Casein

It might be expected that all the casein in a sample of milk would be precipitated by adding sufficient acid to bring the pH value to approximately 4.6. However, acid reacts with the protein complex at a slow rate at this pH value, the pH value will change in the direction of alkalinity during precipitation, and the reaction will not reach completion. If a considerable excess of strong acid, such as hydrochloric, is added, the complex will be broken down more rapidly, but some of the separated casein will dissolve as chloride and may not reprecipitate completely on readjustment of the reaction to the isoelectric point. To stabilize the pH value, a buffer mixture of acetic acid and sodium acetate may be used. In order to increase the rate of precipitation, the acetic acid is added alone to give a pH value slightly on the acid side of the isoelectric point. After a few minutes, the sodium acetate is added and thus the pH value is brought to the isoelectric point and stabilized there. Because of the buffer action of the acetate mixture, the same proportions of acid and salt are equally effective with milks of different percentages of protein complex. Maximal precipitation of casein from milk is effected by adding per each 10 ml of milk, 80 ml of water at 40°C., then 1.0 ml of 10% acetic solution, and, after 10 min., 1.0 ml of normal sodium acetate solution.[180]

Preparation of Casein for Research Purposes

The earliest accepted method for the preparation of so-called "pure" casein was that of Hammarsten.[73] Many modifications of this method and a few somewhat different methods have been offered. Brief reference is made below to some of these, but the original publications should be consulted for precise details. Casein

"nach Hammarsten" is prepared by the following method. Skimmilk is diluted with four times its volume of water and the casein precipitated by the addition of dilute acetic acid. The casein is then repeatedly dissolved in water containing the least amount of alkali that will dissolve it, the solution filtered to remove insoluble substances, the casein reprecipitated with dilute acetic acid and washed with water. The casein must not be exposed to high concentrations of hydroxyl ions nor of hydrogen ions because of the danger of racemization or hydrolysis under these respective conditions. Without previous drying, the casein is triturated with absolute ethanol, the ethanol removed by vacuum, the casein treated with anhydrous ether, the ether removed, and the product dried either over sulfuric acid at reduced pressure or over calcium chloride at atmospheric pressure. The first method of drying yields a product that is difficult to wet with water, the second method one that contains traces of water and is therefore readily wetted.

Robertson[177,178] claimed that Hammarsten casein contains a small amount of a water-soluble, acid substance and, to remove it, he recommended washing the casein with twelve times its weight of distilled water in six portions, followed by five times its weight of absolute ethanol in small portions, and similarly the same volume of ether previously distilled over sodium. Van Slyke *et al.*[219–221] believed that purified casein contained somewhat less phosphorus than the 0.85% characteristic of Hammarsten casein and described several procedures for preparing such a casein. These involve, in one method, a high dilution of the skimmilk used and the use of ammonium oxalate as a precipitant for calcium, and, in another, the removal of calcium and magnesium phosphates by centrifuging from a casein solution at pH 7.0 and electrolysis of a casein suspension at pH 4.7 to remove traces of phosphate. Cohn and Hendry[35] advocated filtration of a casein solution at pH 6.3 as a means of removing calcium caseinate and calcium phosphate. Sandelin[184] described the removal of calcium by means of potassium oxalate added to the skimmilk before precipitation of the casein. Dunn[45] precipitated casein at pH 4.8 by the addition of 0.5 N hydrochloric acid to diluted skimmilk, then washed the precipitate with water, ethanol, and ether.

Warner[225] obtained raw milk at time of milking, added toluene as preservative, and chilled the milk immediately to 2°C. All subsequent operations were carried out at that temperature. The milk was skimmed and the casein was precipitated at pH 4.6 by the addition of 0.1 N hydrochloric acid. It was then washed with ice-

water, dissolved with sodium hydroxide to pH 6.5, and the solution was extracted with ether. The solution was diluted and the casein was reprecipitated with 0.01 N hydrochloric acid. Finally, it was washed thoroughly with water. A dry product was also made using ethanol and ether. This casein contained 0.86% phosphorus.

Precipitation of casein at pH 4.1, as in the commercial grain-curd process described later, followed by thorough washing with slightly acidulated water, removes all but about 0.20% calcium oxide and all of the phosphorus pentoxide in excess of 1.80% (0.79% phosphorus). For most purposes, the small quantity of extraneous ash remaining—less than 0.50%—is not objectionable, and, what is of considerable importance, the casein is not exposed to alkalinities greater than that of milk itself.

Casein that is practically free of both ash and vitamins may be prepared by a method of Block and Howard,[12] which avoids exposure of casein to alkaline pH values. Purified sulfur dioxide is bubbled through skimmilk held at approximately 36°C. until the pH value is 4.5–4.6. The precipitated casein is removed by filtration and washed with water at pH 4.5–4.6. The casein is redissolved by stirring it in suspension in water and passing in sulfur dioxide until the pH value is 1.8–1.9. It is reprecipitated by adding dilute alkali until the pH value is again 4.5–4.6, then separated by filtration, washed with water, and dried by use of anhydrous solvents as described above.

Preparation of Casein for Industrial Use

Commercial casein is made by either of two general methods—precipitation by acid or coagulation by rennet. The rennet method is used almost entirely and exclusively for producing casein for the manufacture of plastics, since rennet casein has the peculiar properties considered essential for this product—properties apparently closely related to its high ash content. Acid precipitation is employed for producing casein for its other commercial uses, for most of which adhesive properties are the most important requirement.

In the manufacture of rennet casein,[145] a fresh, low-fat, skimmilk is warmed to 35.5°C. and curdled with about 4 oz. of rennet extract to each 100 gal. of milk. The coagulation should be complete in 15 to 20 min., after which the curd is broken up and gradually heated to 55 to 65°C. After it has settled for 10 min., the curd is drained of whey and washed several times with water at 26° to 32°C. It is then pressed for an hour, shredded, and dried in thin layers at 43°

to 46°C. Rapid drying at a low temperature is essential if the product is to be of a light color.

Acidification methods[2,3,4] may be of the self-sour type, in which the acid is formed in the skimmilk by bacterial fermentation of lactose to lactic acid, or of the type in which acids are added to the milk in sufficient quantity to precipitate the casein and attain the desired degree of acidity. There are numerous modifications depending on the acid and the temperatures used and on the mechanical equipment. Several procedures employ continuous precipitation, washing and drying, which, as is to be expected, give a product of remarkable uniformity in composition, color, and adhesiveness.

In the self-sour processes, the milk is inoculated with lactic acid bacteria and allowed to stand at a favorable temperature until curdling takes place. The curd at this acidity—about pH 4.7—is soft and fine, and, in order to agglomerate it, heat is employed. Excessively high temperatures produce a rubbery curd that is impossible to wash successfully unless it is chopped. After draining, the curd is washed several times, drained, pressed overnight, shredded, and dried at 54°C. Casein of this type, unless exposed to acidity greater than pH 4.7, contains a somewhat greater percentage of ash than the grain-curd casein described below.

The above-described process may be carried out more rapidly, if the curd is precipitated by the addition of dilute acid with moderate stirring. But, if a mineral acid, such as hydrochloric or sulfuric, is added to the skimmilk at above 35°C. in sufficient quantity to bring the reaction to pH 4.1 (apparently pH 4.6, if methyl red is used as indicator), a curd of coarse, granular testure is formed. This is known as grain-curd and, because of its open texture and the fact that calcium phosphate is completely in solution at this acidity, it is easily washed to produce a low-ash, low-acid casein. In the continuous adaptation of this process, temperatures of coagulation as high as 43°C. are employed and the chewing-gum textured curd produced is chopped before washing. The washing, pressing, shredding, and drying operations are the same for all types of curd. Casein for paper-coating should be ground to 20-30 mesh, the proportion of finer particles being kept as small as possible.

Uses of Casein

The most extensive use of casein in the United States is in coating paper for books and magazines. For this use it is dissolved in alkali, mineral pigments are added, and the suspension is applied to

the paper by means of rollers or brushes. The casein binds the pigment to the surface of the paper and renders the surface smooth and nonabsorptive, thus making the paper suitable for fine printing. Formaldehyde may be used to make the casein-bound coatings waterproof, as on playing cards and wall papers. Casein is used in the paper industry also as a dispersing agent for the rosin used for sizing paper.

Casein glues are used chiefly in the woodworking industries. They consist of casein, a solution of alkali as solvent and a calcium compound. The calcium caseinate formed becomes insoluble on drying and causes the glue to become waterproof.

Ground casein can be converted into fibrous forms by extruding an alkaline solution of the protein into an acid coagulating bath, or by extruding a heated mixture of casein and water into air. The term casein fiber is reserved for the fine wool-like filaments obtained by the first method. The coarser product of the second method is called casein bristle. Casein fiber was produced commercially in the United States for a number of years as a wool substitute during war-time scarcity. As an example of the research which was carried out with the goal of improving the properties of the fiber, the description of continuous-filament casein yarn by Peterson *et al.* in 1948 may be cited.[165] The preparation of casein bristles was reported by McMeekin *et al.*[81,129,164] in 1945 and these, too attained limited commercial production.

Plastic casein is made by stirring dry rennet casein, pigments, and a small proportion of water into a heavy dough and extruding this dough through dies under pressure. Formaldehyde is used as an insolubilizer and hardening agent, being applied usually to the finished article.

Casein-containing, water-thinned paints may consist of pigments, an insolubilizer and a solution of casein in alkali, or may contain oil in the form of an oil-in-water emulsion. The first type, of which whitewash made from skimmilk and lime is the simplest example, is used mostly on exterior surfaces; the second type is popular for use on interior walls and ceilings.

Casein is used in the textile industry for many purposes, such as fixing colors, loading, sizing, softening, and waterproofing. In the leather industry it is used in solution in a minimum amount of alkali to give a gloss to light leathers. A mixture of lime and casein is used as an adhesive and spreader in applying insectides. Many more uses of minor importance are listed by Sutermeister and Browne.[203]

Fractions of Casein

It has been realized for some time that although casein may be prepared and purified in a readily reproducible manner, the product cannot be regarded, chemically, as a single protein; rather, it is made up of a number of different proteins which can be separated and shown to have distinctive properties. The constancy in composition of the mixture of proteins which constitute casein is remarkable and must reflect the strength of the bonds which hold the different components together under ordinary circumstances. At the same time, it is possible to find certain conditions where the bonds can be broken and the mixture dissociated without harm to the individual components. These conditions provide the bases for the various methods for fractionating casein.

Casein has been fractionated on the basis of differential solubility in solutions of hydrochloric acid,[119] acidified ethanol,[118] and 50% ethanol,[153] by precipitation from solutions containing ammonium chloride by means of acetone,[29,30] and by successive precipitations by other methods.[67,93] Fractions of varying phosphorus, tyrosine, and tryptophan content were obtained but in these earlier investigations no evidence for homogeneity of the fractions was presented, nor was complete separation into distinct components claimed. The newly developed electrophoretic method of Tiselius was applied to the problem by Mellander in 1939. The method not only revealed the presence of three components in casein but enabled Mellander to prepare small amounts of two, α- and γ-caseins, which were found to be quite different in their phosphorus to nitrogen ratios.[132] The electrophoretic method was also important subsequently in guiding the development of chemical methods for the fractionation of casein and the preparation of its components in quantity.

The separation of casein into α- and β-caseins, its principal components, was achieved by Warner[225] by preparing a very dilute solution of acid-precipitated casein, 0.2 to 0.3%, at pH 3.5 and 2° and then adding 0.01 N sodium hydroxide to pH 4.4. Under these conditions β-casein is more soluble and largely remains in solution, whereas α-casein is precipitated. By reprecipitating the α-casein in a similar manner at least six times it is obtained free of β-casein as shown by electrophoresis. From the supernatant solutions β-casein is precipitated by adjusting the pH to 4.9 at room temperatures. It too can be readily purified so as to be free of other electrophoretic components. Warner's preparations were not electro-

horetically homogeneous under all conditions but neither component contained any of the other.

Two other methods for separating α- and β-caseins and for the preparation of γ-casein as well have been described by Hipp *et al.* In the first[79,80] the fractionation is carried out with solutions of casein in 50% ethanol by varying pH, ionic strength and temperature. In the second[80] casein is dissolved in 6.6*M* urea and fractionation is accomplished by the addition of water.

The development of chemical methods for the preparation of the principal electrophoretic components of casein has enabled numerous investigators to study their properties and composition. A few of the distinctive properties are listed in Table 21, (p. 56,) and the amino acid compositions are presented in Table 23 (p. 60).

A fourth fraction was isolated from casein by Cherbuliez *et al.*[27,28] and was named Δ-casein. There are some similarities, however, between Δ-casein and Hammarsten's proteose,[1,27] a substance found after casein is treated with rennet, and at present, Δ-casein is not classified as a component of casein.[19]

That α-casein is not always homogeneous in its electrophoretic behavior has already been mentioned. In 1955 and in subsequent publications, Von Hippel and Waugh[86,226,228] described the separation of casein from milk by high-speed centrifugation and its fractionation by means of calcium ions. They showed that the α-casein fraction was really made up of sub-fractions: α_s-casein, precipitable by calcium ions under certain conditions and also called "calcium-sensitive casein;" crude κ-casein (calcium-insensitive casein) not precipitable by calcium ions and containing a sub-fraction designated *m*-fraction. The importance of the various components in interactions which result in the stable casein micelles of milk has been stressed by Waugh. In his most recent paper,[227] published in 1961, it is stated that three sub-fractions of α-casein, now designated α_s1-, α_s2-, and κ-casein, have been isolated in pure form and characterized, but experimental details are not yet reported.

The heterogeneity of the α-casein fraction has also been investigated by McMeekin *et al.*[78,84,126] In this work, a well-washed, acid-precipitated casein was further purified by extraction with dilute acetic acid at pH 4.0. Incidentally, from this extract McMeekin has prepared a fraction, named α_z-casein, which has marked activity in stabilizing "calcium-sensitive" casein fractions.[125] The purified casein was then fractionated by a modification of Warner's method to give α-casein, which was, in turn, fractionated by means of calcium ions to yield "calcium-insoluble" and "calcium-soluble"

sub-fractions. The "calcium-insoluble" α-casein was further pu fied by extractions with ammonium sulfate to give the principal component of α-casein, as shown by electrophoresis in acid solutions, which was designated α_1-casein.[126] From the "calcium,-soluble" α-casein two other electrophoretic components, α_2- and α_3-caseins, were prepared by the use of the ultracentrifuge.[78,84] The names of components in these investigations were assigned on the basis of electrophoretic mobility at pH 2.35, α_1-casein migrating most rapidly under these conditions; the three components have approximately the same mobility, that of α-casein, in alkaline solutions. In many properties α_1-casein is similar to Waugh's α_s-casein, α_3-casein is apparently related to κ-casein, and α_2-casein resembles λ-casein, a fraction studied by Long, Van Winkle, and Gould.[122] Some of the properties of components of α-casein were reviewed and summarized by Brunner *et al.* in 1960[19] and amino acid analyses of α_1-, α_2-, and α_3-caseins may be found in the report of Hipp *et al.*[78]

It is evident from the preceding paragraphs that whole, acid-precipitated casein, simple though it may seem when compared with the casein complex in milk, is, in itself, an extraordinarily complicated chemical mixture. Perhaps the best picture of its complexity may be obtained from the analyses by zone electrophoresis in concentrated urea solutions described by Wake and Baldwin.[224] These authors report that about 20 components can be resolved in a single starch-gel analysis of casein, most of the new components being found in the alpha fraction. Still another aspect of the complexity of casein has been brought to light by Aschaffenburg.[4] Caseins were prepared by isoelectric precipitation from the milks of individual cows and inspected by the technique of paper electrophoresis in buffers containing urea. Evidence was obtained for the existence of gene-controlled variants of the β- and γ-components of casein. Aschaffenburg concludes that several independent genes are involved in the elaboration of casein by the mammary gland and that the classic division of casein into α-, β-, and γ-components is justified and meaningful.

WHEY PROTEINS

Prior to 1934, it was generally considered that whey protein consisted chiefly of two definite substances, a lactalbumin fraction and a lactoglobulin fraction, and of comparatively insignificant amounts of several other substances of the nature of proteins. But, in 1934, Palmer[155] reported the isolation of a protein having the char-

eristics of a globulin from the lactalbumin fraction. This protein, which comprises more than half of the lactalbumin fraction, was at first called Palmer's globulin, but is now named β-lactoglobulin. This and several other proteins have now been isolated from the classical fractions by methods to be described.

Preparation of Whey Protein

The proteins of cheese whey, which include some casein in addition to globulins and albumins, may be precipitated together and recovered efficiently by procedures that require careful control of temperature and acidity.[23,24,130,229] If a product free from casein is desired, the whey should be from the acid precipitation of casein from skimmilk as previously described. The whey is put through a cream separator to remove as much fat as possible, its reaction adjusted to pH 6.3–6.5 by addition of 10 N sodium hydroxide, and the whey heated to above 90°C. with constant stirring to render the protein precipitable by acid. Then, 100 ml of 33% acetic acid is added rapidly for each 100 lb. of whey and the stirring stopped as soon as the acid is thoroughly distributed. When the coagulum has collected, it is removed by filtration and, if desired, washed, drained, and dried. Solutions of other acids may be employed as precipitants in place of the acetic acid, the reaction being brought to pH 4.8–5.3. The whey protein prepared in this way is suitable for use in food products.

Other methods for the preparation of whey proteins in both denatured and undenatured forms have been proposed. Some of these have been described by Whittier and Webb.[235] Block *et al.* used ferric chloride to precipitate whey proteins as iron derivatives,[11] collectively called "ferrilactin," from which the iron could be removed subsequently, if desired.[14]

Preparation of the Lactoglobulin Fraction

The lactoglobulin fraction is isolated by saturating skimmilk with sodium chloride to precipitate casein, filtering to remove the casein, heating the filtrate to 35°C., filtering to remove any precipitate formed, and finally saturating the solution with magnesium sulfate.[187] Rowland[180] obtained complete precipitation of the lactoglobulin fraction by removing casein from milk by means of acetic acid and sodium acetate as described earlier in this chapter, adjusting the reaction to pH 6.8–7.2, saturating the liquid with magnesium sulfate, and allowing the mixture to stand for a few hours.

Preparation of the Lactalbumin Fraction

The so-called lactalbumin fraction may be prepared[187] by saturating skimmilk with magnesium sulfate, removing the precipitated casein and lactoglobulin fractions by filtering, and adding 0.25% acetic acid to the filtrate until a permanent turbidity is produced. After some time, the precipitate is removed by filtering, redissolved in water, the solution neutralized, and again saturated with magnesium sulfate and treated with acetic acid. This procedure is repeated from 3 to 6 times.

The method employed by Sjögren and Svedberg[189] consists of half-saturating skimmilk with ammonium sulfate, adding acetic acid to give a pH value of 5.2, removing the precipitated casein and lactoglobulin fractions by filtration, and increasing the ammonium sulfate concentration to 80% of saturation. The resulting precipitate is removed by centrifuging, dissolved in water, and reprecipitated by adding ammonium sulfate and sulfuric acid. It is then dissolved in water, the solution electrodialyzed, the protein precipitated by adding ethanol, filtered, washed with ether, and dried.

β-Lactoglobulin

In 1934 Palmer[155] discovered that a crystalline protein, insoluble in water, could be prepared from the classical lactalbumin fraction. In the following years the protein was named β-lactoglobulin, it was shown to be the most abundant of the whey proteins, it was widely used in protein chemistry in research requiring a pure, crystalline protein, and it was used for the first complete amino acid analysis of a fairly large protein. The extensive literature concerning the chemical and physical properties of this protein was summarized by Tilley[209] in 1960 in a comprehensive review.

In Palmer's original method for preparing β-lactoglobulin, casein was removed from skimmilk by adding hydrochloric acid to pH 4.6. The pH of the whey was then adjusted to 6.0 and ammonium sulfate added to half-saturation. The precipitate was removed and discarded. The filtrate was then saturated with ammonium sulfate, the precipitated protein removed, and dissolved in water. On long dialysis of the solution at pH 5.2, an oil appeared which was gradually transformed into crystals. Palmer described also a "routine" method in which whey, adjusted to pH 5.8, was frozen to a solid block, and a concentrate comprising about one-fourth of the original volume was collected by slow thawing of the block in a cold room. Addition of 18 gm. of anhydrous sodium sulfate per each 100 ml of

ncentrate caused the formation of a precipitate, which was removed after a few hours and discarded. To the filtrate, warmed to 30°C., was added another 18 gm. of anhydrous sodium sulfate per 100 ml. When all the salt had dissolved, the precipitate was removed by filtration in a warm room, scraped from the filter paper, and dissolved in sufficient water to give a solution containing 8–10% protein. The solution was cooled to 0°C. and precipitated salt was removed and washed. The solution and washings were combined, the pH was adjusted to 5.8–6.0, toluene was added, and the solution was dialyzed against distilled water for 7 days. Some precipitate which had formed was removed by filtration and the filtrate was adjusted to 5.2 by means of hydrochloric acid, whereupon crystallization ensued. The crystalline protein was practically insoluble in water at or near pH 5.2, but it was easily soluble in dilute salt solutions from which it could be recrystallized by dialysis.

Palmer's original method for preparing β-lactoglobulin was modified by Sørensen and Sørensen[197] in that casein was removed at the pH of milk by means of ammonium sulfate, and this and other modifications were proposed in a method by Cecil and Ogston.[26a] A detailed procedure based on the Sørensen modification has been described also by Larson and Jenness.[105] Aschaffenburg and Drewry[8] prepared β-lactoglobulin in a somewhat different manner. In their method casein, globulins, other protein and fat are precipitated together from whole milk by means of sodium sulfate. The precipitate is removed and the filtrate is acidified. Under these conditions α-lactalbumin precipitates and is removed, but lactoglobulin does not. The latter is salted out from the filtrate by neutralization to pH 6 and the addition of more sodium sulfate and is then crystallized by dialysis. The last step is relatively rapid in the absence of α-lactalbumin.

That even well-purified β-lactoglobulin, crystallized and recrystallized many times, evidently a single substance in both electrophoretic and ultracentrifugal behavior,[159] was not truly homogeneous, became apparent from the solubility studies of Grönwall[68] and the electrophoretic experiments in acetate buffers by Li[115] and McMeekin *et al.*[128] The fractionation of crystalline β-lactoglobulin and the isolation of a homogeneous crystalline component, designated β_1-lactoglobulin, were accomplished by Polis *et al.*[170] in 1950. Fuller understanding of the heterogeneity came with the discovery by Aschaffenburg and Drewry[5] that individual cows produce either a mixture of two electrophoretically distinct β-lactoglobulins, or only one or the other of these. Although at first called β_1- and β_2-lacto-

globulins, the correlation of these with Polis' components was r entirely clear and subsequently, when Aschaffenburg and Drewry found that the capacity to produce the different types of β-lactoglobulin was genetically controlled,[6] the two forms of the protein became known as β-lactoglobulins A and B.

Since it has been shown that the two forms of β-lactoglobulin are so closely related in physical properties and chemical composition, most of the numerous data that were obtained through use of the well-purified, crystalline protein from mixed herd milk are no doubt valid and useful. The small differences in properties—in titration curves,[207] electrophoretic mobilities,[150,210] and molecular weights under certain conditions[211]—were summarized by Townend *et al.* in a paper[212] which also presented new evidence for a difference in primary structure of the proteins. By amino acid analysis, Gordon *et al.*[54] and Piez *et al.*[167] found that the only differences in composition were that a molecule of β-lactoglobulin A contained two more residues of aspartic acid and valine and two fewer residues of glycine and alanine than β-lactoglobulin B. The results of analyses of β-lactoglobulin AB, prepared from mixed herd milk are presented in Table 23 (p. 60).

β-Lactoglobulin is a simple protein containing only amino acids. It is unlike casein also in that it contains free sulfhydryl groups in the form of cysteine residues. These have been implicated in the development of "cooked" flavor when milk is heated[90,95,104]

α-Lactalbumin

α-Lactalbumin, second in concentration to β-lactoglobulin among the whey proteins, can also be crystallized from the lactalbumin fraction. The name was first used in connection with a "lactalbumin" isolated by Kekwick and its behavior in the ultracentrifugal investigations of Svedberg and Pedersen.[204] It is most likely that the same protein was crystallized by Sørensen and Sørensen[197] in 1939 and designated "crystalline insoluble substance" because of its insolubility in water at pH 4.6. A method for the isolation of the protein, based on the observations of Sørensen and Sørensen, was reported by Gordon and Semmett[58] who also characterized the protein and proposed that it be known as α-lactalbumin. The method was later modified[62] and described in detail.[64] In principle, the original Palmer method is used to remove casein and the lactoglobulin fraction from skimmilk and to crystallize β-lactoglobulin from the lactalbumin fraction. The mother liquor from the crystallization is adjusted to pH 4 and ammonium sulfate is added

precipitate crude α-lactalbumin. It is dissolved at pH 8, reecipitated at pH 4, dissolved again, and crystallized by the addition of ammonium sulfate to 2/3 saturation at pH 6.6; it is recrystallized by repeating these steps.

Two other methods for the preparation of α-lactalbumin have been reported. Zweig and Block[241] precipitated the whey proteins as iron derivatives, "ferrilactin," removed iron by means of an ion-exchange resin and dialyzed the acid, iron-free solution of proteins. When the dialyzed solution was adjusted to pH 5.2, a precipitate formed. The washed precipitate yielded α-lactalbumin, while the mother liquor could be fractionated to give both β-lactoglobulin and more α-lactalbumin. The method of Aschaffenburg and Drewry[8] has been mentioned previously in connection with the preparation of β-lactoglobulin. The precipitate which is formed on acidification to pH 2 of a crude lactalbumin solution containing sodium sulfate, consists almost entirely of α-lactalbumin. It is reprecipitated several times by acidification of weakly ammoniacal solutions and then crystallized by means of ammonium sulfate.

A few of the outstanding properties of α-lactalbumin are shown in Table 21 (p. 56). Its unique amino acid composition is given in Table 23 (p. 60); α-lactalbumin contains no free sulfhydryl groups though its content of cystine is high. There is some evidence, as yet inconclusive, that this simple protein, too, may be heterogeneous in mixed herd milk and that the occurrence of two forms may be genetically determined.[17]

Lactoglobulins (Immune Lactoglobulins)

These proteins are present in ordinary milk in small concentration but they occur in colostrum—the milk secreted for a few days after parturition—in much larger amounts. They are of unique importance to the new-born calf because they are absorbed into its circulation, where they fulfill, temporarily, the immunological functions of blood gamma globulin. References to papers on this subject have been summarized by McMeekin.[124]

The lactoglobulin fraction of whey prepared by saturation with magnesium sulfate[187] contains both euglobulin, insoluble in water, and pseudoglobulin, soluble in water[39]; other proteins are present also, as shown by Smith.[192,193] To prepare purified eu- and pseudoglobulins Smith precipitated the lactoglobulin fraction from acid whey by half-saturation with ammonium sulfate. The precipitate was dissolved, the solution adjusted to pH 4.6 and ammonium sulfate added to one-fourth saturation. A precipitate, which contains

casein, was removed. The globulins were then precipitated at pH 6.0 by the addition of ammonium sulfate to 0.4 saturation. After another purification step the globulins are separated into euglobulin and pseudoglobulin by exhaustive dialysis. The differences in electrophoretic mobilities, sedimentation constants, etc., reported by Smith for the two globulins are not great and he found that they are also closely related in chemical composition. For example, both contain about 2.5 to 3.0% hexose and 1.3% hexosamine; other data are listed in Tables 21 (p. 56) and 23 (p. 60). The differentiation into eu- and pseudoglobulin components is, therefore, not very meaningful. Moreover, it is probable that the immune globulins of milk are identical with those of colostrum[192] and of bovine blood.[103,124,192]

The use of rivanol (6,9-diamino-2-ethoxyacridine lactate) for the isolation of immune globulins from milk and colostrum has been described by Kenyon *et al.*[99]

Blood Serum Albumin

By repeated fractionations of the mother liquor remaining after crystallization of β-lactoglobulin from the crude lactalbumin fraction, Polis *et al.*[169] succeeded in isolating and crystallizing a true, water-soluble milk albumin. When this was compared with crystalline bovine serum albumin, it was found that the two were identical in physical properties and composition; some of these data are shown in Tables 21 (p. 56) and 23 (p. 60). It was demonstrated also by Coulson and Stevens that the proteins were immunologically indistinguishable.[37]

Other Milk Proteins

It has been mentioned elsewhere in this chapter that a proteose-peptone fraction, amounting to about 2–6% of the total protein, is present in milk. Various workers have prepared substances of this nature from milk under such names as minor-protein fraction, sigma-proteose, and milk component 5, as well as proteose-peptone. These substances, and also a fat globule membrane protein isolated from cream, have been investigated and compared by Brunner and Thompson.[20] Their paper may be consulted for references to earlier work on these proteins.

Many types of enzymatic activity have been demonstrated in milk. In some instances it has been possible to isolate and crystallize the pure enzyme from milk, as Avis *et al.*[9] have done with xanthine oxidase and Polis and Shmukler[168] with lactoperoxidase.

A minor protein, which is red when combined with iron ions and colorless when free of iron, has been isolated and characterized by Groves.[69] It is a glycoprotein which combines with two atoms of iron per molecule of protein to form a red compound.

These and many other proteins occur in milk in small amounts. Summaries of the literature on minor proteins in milk can be found in the chapter by McMeekin[124] and in a report by Whitney.[233]

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